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Tomato Spotted Wilt *Tospovirus* Genome Reassortment and Genome Segment-Specific Adaptation

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A system to associate specific genome segments with viral phenotypes and to study factors influencing genome reassortment was developed for tomato spotted wilt *Tospovirus* (TSWV). Reassortant isolates were generated by co-inoculating a TSWV isolate, TSWV-D, with TSWV-10 or TSWV-MD. The parental origin of each genome segment in putative reassortant isolates was determined by segment-specific restriction fragment length polymorphisms. The TSWV isolates readily exchanged genome segments in a nonrandom fashion. The S RNA from TSWV-D was dominant over the S RNA from TSWV-10. The intergenic region (IGR) of the S RNA was correlated with competitiveness of this genome segment in reassortant isolates. The less competitive S RNA contained a net increase of 62 nt, including a 33-nt duplication in the IGR. This duplicate sequence was highly conserved among isolates from the southeastern United States and an isolate from Bulgaria. The evidence supports the hypothesis that the IGR of the S RNA with an ambisense coding strategy serves a regulatory function which influences the occurrence of this segment in the viral population. In addition, it was demonstrated that stable parental phenotypes can be mapped to specific genome segments as well as generating novel phenotypes not associated with either parent.

INTRODUCTION

Tomato spotted wilt *Tospovirus* (TSWV) is the type member of the genus *Tospovirus* in the family *Bunyaviridae.* The absence of either protocols for reverse genetics or a system for classical genetic mapping is a limiting factor which impedes progress in increasing our understanding of the pathogenicity of this economically important virus. Viruses classified in the *Bunyaviridae* are characterized by having quasi-spherical, enveloped virions packaging a tripartite, RNA genome (Murphy *et al.,* 1995). Genera are distinguished by their genome organization and the translational polarity of the genome segments, which are in either negative-sense or a combination of negative-sense and ambisense. The tripartite genome organization of the *Bunyaviridae* family offers the potential for the viral genomes of closely related viruses to exchange genetic information by reassortment as a mechanism of generating diversity. The TSWV genome consists of three single-stranded RNA molecules labeled as L RNA, M RNA, and S RNA (reviewed by German *et al.,* 1992). The L RNA possesses a single open reading frame (ORF) in the viral complementary sense, which encodes a putative RNA-dependent RNA polymerase (RdRp, 331.5 kDa) (de Haan *et al.,* 1991). The RdRp purified from TSWV virions has been shown to have a replicase function (Adkins *et al.,* 1995). The ambisense coding strategy of the tospovirus M RNA was first demonstrated for impatiens necrotic spot *Tospovirus* (Law *et al.,* 1992) and later confirmed for TSWV (Kormelink *et al.,* 1992). The M RNA encodes a nonstructural protein (33.6 kDa) in an ORF near the 5′ terminus of the viral sense RNA and the G1/G2 glycoprotein (127.4 kDa) in an ORF in the viral complementary sense located near the 3′ terminus of the viral RNA. Both ORFs are translated from subgenomic RNAs. The S RNA also has an ambisense coding strategy with two ORFs: a nonstructural protein (NSs, 52.4 kDa) of unknown function is encoded in a viral sense ORF from the 5′ region of the viral RNA and the nucleocapsid protein (N, 28.8 kDa) is encoded in an ORF in the viral complementary sense near the 3′ terminus of the viral RNA (de Haan *et al.,* 1990). Both the S RNA and M RNA possess an intergenic region (IGR) with a characteristically high A-U content for which no definite functions have been linked. Our goal was to develop a genetic system that could be used to map phenotypes to specific segments of the viral genome and to identify factors which influence reassortment and adaptation.

While the extreme adaptability of this virus to resistant hosts has been recognized for many years, little progress has been made in understanding the genetics of TSWV (Best and Gallus, 1953; Finlay, 1952, 1953). Best (1961) observed the generation of isolates which pos-

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sessed the phenotypes of both parental isolates, which he attributed to recombination. Recently progress has been reported toward development of a system for rescue of transcripts of viral cDNA of Bunyamwera *Bunyavirus* (Bridgen and Elliott, 1996). This system remains highly inefficient and has not yet linked a function to a specific ORF. Although there has been no genetic investigation linking viral phenotypes to the genome segments for any members of the genus *Tospovirus,* phenotype mapping studies in the genus *Bunyavirus* have provided insight into the determinants for insect transmission (Beaty and Bishop, 1988) and infectivity neutralization (Gentsch *et al.,* 1980).

We described in this report a system for the genetic analysis of TSWV and identified elements in the IGR that are associated with competition between genome segments from different parental isolates. We also demonstrated the utility of this system for mapping several phenotypes to specific viral genome segments. A novel viral phenotype, not associated with either parent, was also generated by a specific combination of parental genome segments.

RESULTS

TSWV reassortants

Requisite to a molecular genetic analysis of TSWV is a system consisting of diverse isolates that readily reassort genome segments, for which the genetic markers are available to determine the parental origin of each genome segment in progeny isolates. TSWV-D was initially selected as the universal parent because it was serologically distinct from most other TSWV isolates based on differential reaction with a panel of monoclonal antibodies (unpublished data), but had a wild-type host range and induced typical TSWV symptoms. The specific regions on each genome segment were selected and amplified by RT-PCR (Fig. 1). One marker from each of the two ORFs on the M and S RNAs was assayed for potential recombination of whole ORFs among the genome segments. In addition, a central region of the L RNA was chosen for the primary marker on that segment because this region was absent from defective interfering RNAs (DIs), reducing the possibility of artifacts coincident to the generation of DIs. Numerous amplifications and subsequent digestions corroborated the fidelity of the molecular signature for each isolate.

A restriction fragment length polymorphism (RFLP) of the RT-PCR-amplified products from TSWV-D, TSWV-10, and three reassortants (R1–R3) exemplifies the utility of this system for following the reassortment of TSWV genome segments (Fig. 2). Genetic marker analysis additionally indicated that intermolecular recombination of complete ORFs on the M and S RNA segments or the two RT-PCR-amplified regions on L segments in reassortants did not occur. Initially some reassortants (data not shown) contained an asymmetric complement of genome segments that appeared as one or more segments contributed by both parents with the remaining segments contributed by only one parent (e.g., $L_{10}M_DS_{10+D}$). These isolates were discarded or were continually transferred until only one parental type of each genome segment was detected. It is not known if these reassortants were mixed cultures or if a single isolate packaged a mixture of genome segments.

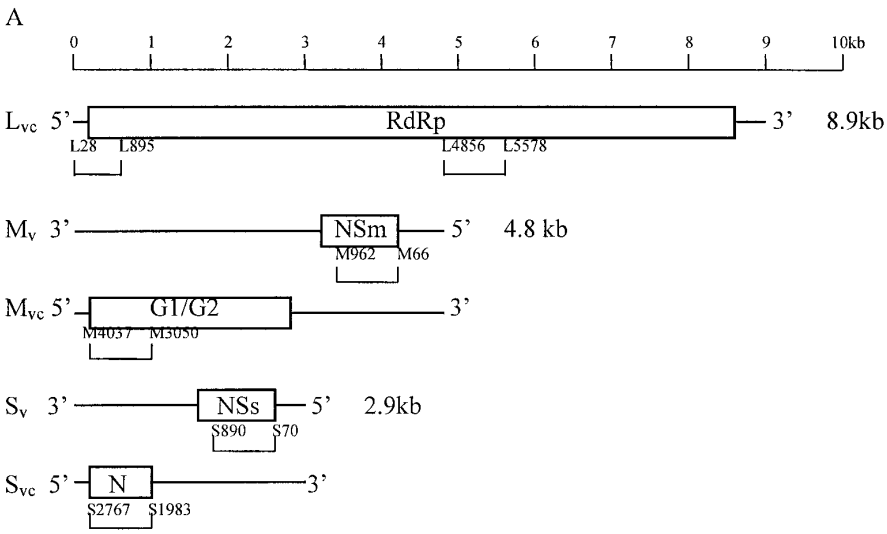
Reassortment frequency

TSWV-D was co-inoculated with two other TSWV isolates in separate experiments (TSWV-D $\times$ TSWV-10) to determine the ability of each TSWV-D genome segment to reassort with those of other isolates and to determine the frequency with which the possible combinations of genome segments from the parental isolates would be distributed into reassortants. By χ^2 analysis, the frequency of detection of the parental and reassortant genotypes was observed to be nonrandom. In one experiment the two parental isolates and three reassortant genotypes ($L_{10}M_{10}S_D$, $L_{10}M_DS_D$, and $L_DM_{10}S_{D)}$ were identified from the analysis of 58 progeny isolates (Table 1). Forty progeny isolates were reassortants and the remaining 18 isolates represented parental types. $L_{10}M_DS_D$ was the dominant reassortant genotype, representing over half of the progeny isolates recovered. TSWV-D was the next most frequent genotype. The L segment was contributed by TSWV-10 in 72% of the progeny isolates and TSWV-D contributed the M segment in 79% of the progeny isolates. TSWV-D contributed the S segment to all of the reassortants. Two repetitions of this experiment were conducted, and in each case, only the S segment from TSWV-D was detected in the progeny reassortants. No other statistically significant patterns of assortment or co-assortment of two segments were detected.

Replication divergence of S RNA segments

To further investigate the basis for the competitive advantage of the S segment from TSWV-D, full-length cDNAs of the S RNAs from TSWV-D and TSWV-10 were sequenced. There was 98, 94, 84, 97, and 98% sequence identity for the 5′ untranslated region (UTR), NSs, IGR, N, and 3′ UTR domains between two S RNAs in the viral sense, respectively. While the overall identity between the two S RNAs was 94%, there was a net increase of 62 nt in the length of the S RNA from TSWV-10. The difference in length was attributed solely to the IGR (Fig. 3). The sequence length difference was also confirmed by amplifying the IGR fragments directly from the total RNA with primers S2083 and S1457 and resolving the products on agarose gels (data not shown).

The increased length of TSWV-10 S RNA could be attributed to randomly inserted nucleotides and insertion of over 100 nt that contained a 33-nt duplication of se-



L28	GCAAACATGAACATCCAG	Vc
L895	CTGTGGGTGTGGTTCCAAC	V
L4856	CGGGATCCCGCTGTGCAGGTACAAGC	Vc
L5578	CCATCGATGGTGTACATGAACAGCTGA	V
M66	CACAAGCTCCTCTACCTTAGGC	V
M962	CCTCTTCCTCTTCAACTGATCTCTC	Vc
M3050	CCTGTATAATCCGAAAACCC	V
M4037	CCTATACTGTAAGCATCACTAGCC	Vc
S70	CACAGTACCAATAACC	V
S890	CATCTCCTGCAACCTTGAAC	Vc
S1983	CCCTCGAGGCTTTCAAGCAAGTTCTGCG	V
S2767	GCTCTAGAGCCATCATGTCTAAGGTTAAGCTCAC	Vc

B

HNEF	AAGCTTGCGGCCGCGATATC	(for terminal sequence)	
HNER	GATATCGCGGCCGCAAGCTT	(for terminal sequence)	
S156R	CCAGATGCAGTTGATCC	(for terminal sequence)	Vc
S2500	GCTCCAATCCTATCTGAAG	(for terminal sequence)	V
S2083	GGAAGTATTGCTATGG	(for IGR cloning)	Vc
S1282	CACTTGAATGTCTTCC	(for IGR cloning)	V
S1457	CGTATGCTTCAGGATC	(for IGR cloning)	V
SIGR1	GCTTGTGCTTGCTTAGTGTG	(for partial IGR amplification)	Vc
SIGR2	CCCGAAAGGGACAATTTTGGCC	(for partial IGR amplification)	V

FIG. 1 TSWV genome map, coordinates sequences of oligonucleotide primers used for RT-PCR amplification of genome segments. (A) Genome organization of the L, M, and S TSWV genome segments. Both viral (v) and viral complementary (vc) polarities of the ambisense segments are shown. The regions of individual segments used to type genomic reassortment patterns are identified by coordinates on the genome segments. Sequence coordinates are presented in the viral polarity as published for the L (de Haan, 1991), M (Kormelink *et al.,* 1992), and S (de Haan *et al.,* 1990) segments. (B) Primer pairs used to determine the nucleotide sequence of S RNA termini and for analysis of the intergenic region (IGR).

quence found near the 3′ terminus of the IGR that was not present in any of the IGRs from isolates with "short" IGRs. The alignment of TSWV-D and TSWV-10 IGRs revealed other small insertions and deletions, resulting in a net increase of 62 nt in the TSWV-10 IGR. To determine the level of conservation of the variation in the IGR among isolates, the IGRs of eight more isolates from the southeastern United States, California, Hawaii and Europe as well as of three published S RNA sequences (de Haan *et al.,* 1990; Maiss *et al.,* 1991; Pang *et al.,* 1992) were aligned (Table 2). The S RNAs of seven isolates from California, Hawaii, The Netherlands, and Brazil possessed the shorter IGRs and lacked the 33-nt duplicate sequence. In contrast, the S RNAs of five isolates from the southeastern USA and one isolate from Bulgaria had the longer IGRs containing the 33-nt duplication. The

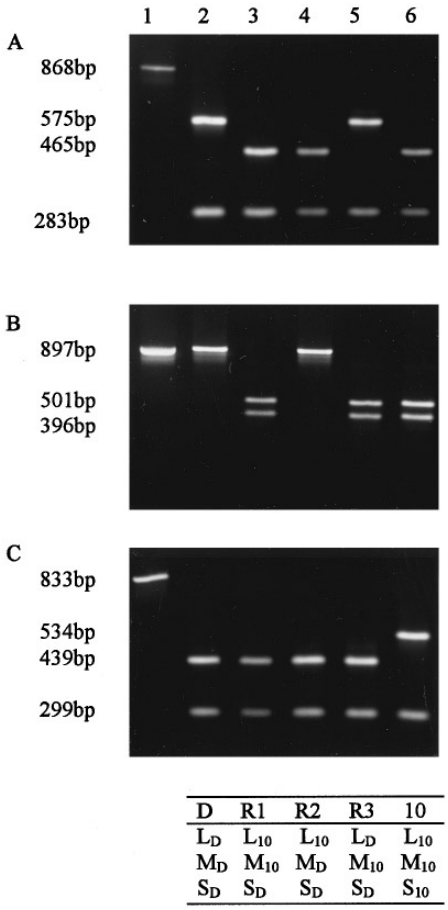


FIG. 2. Restriction fragment length polymorphisms of the amplified fragments used to determine the genotypes of parental isolates, TSWV-D and TSWV-10, and progeny reassortants. (A) Amplified fragments from nt 28 to 895 of L RNA digested by *Taq* I; (B) amplified fragments from nt 66 to 962 of M RNA digested by *Rsa*I; (C) amplified fragments from nt 70 to 890 of S RNA digested by *Taq*I. Lane 1, undigested RT-PCR product; lane 2, TSWV-D; lane 3, reassortant $L_{10}M_{10}S_D$; lane 4, reassortant $L_{10}M_DS_D$; lane 5, reassortant $L_DM_{10}S_D$; lane 6, TSWV-10. The genotype of each isolate is presented below the lane for that isolate.

most conserved regions in the IGRs among all TSWV isolates were the termini of the IGR adjacent to the flanking ORFs.

To investigate the role of the IGR in the S RNA replication, we co-inoculated *Nicotiania benthamiana* plants with another pair of isolates, TSWV-HR2 (short S RNA IGR) and TSWV-10 (long S RNA IGR). The resultant viral population was subjected to seven serial passages on *N. benthamiana* plants, and the isolate-specific S RNA markers in the IGRs were utilized to monitor the relative concentration of the two S RNAs in the viral population. TSWV-HR2 S RNA became increasingly dominant in the viral S RNA pool with each successive passage of the viral mixture. TSWV-10 S RNA diminished until it was not detectable after the seventh passage. Using the L RNA marker as an internal control, we demonstrated that the competitive attribute was segment-specific (Fig. 4). The same trend was also observed in the same viral population subjected to repeated serial passages on *N. tabacum* cv. 'Burley 21' Following multiple passages of another mixed viral population consisting of the paired isolates, TSWV-34 (short S RNA IGR) and TSWV-10, the S RNA marker assay further confirmed that the S RNA segment without the duplicate sequence dominated the viral S RNA population following seven passages on *N. benthamiana* plants.

Correlation of TSWV genome segments with phenotypes

The host responses of parental isolates and reassortants were compared. Symptoms on *Gomphrena globosa, Nicotiania glutinosa, Capsicum annuum, Lycopersicon esculentum, Emilia sonchifolia,* and *N. benthamiana* plants remained similar for both parental isolates and reassortants. However, distinguishable characters, such as lesion morphology and systemic movement, on some host plants made it feasible to relate a particular phenotype to a genome segment (Table 3). TSWV-D produced smaller local lesions (0.3 mm diameter) than TSWV-10 (1.2 mm) on *Cucumis sativus* cv. 'National Pickling' cotyledons. The $L_{10}M_{10}S_D$ and $L_DM_{10}S_D$ reassortants, both acquiring the M RNA from TSWV-10, generated the larger chlorotic local lesions on *C. sativus* cv. 'National Pickling' cotyledons. The $L_{10}M_DS_D$ reassortant, acquiring the M RNA segment from the TSWV-D parent, also produced the smaller chlorotic local lesions. Similar results were observed with the systemic movement phenotype. TSWV-D was capable of systemically invading *N. tabacum* cv. 'Burley 21' plants while TSWV-MD was restricted to local infection. The reassortant, $L_DM_{MD}S_{MD}$, could systemically infect *N. tabacum* cv. 'Burley 21' plants, similar to the parental isolate TSWV-D. Another reassortant, $L_{MD}M_DS_{MD}$, with the L RNA from TSWV-MD, possessed the same phenotype as TSWV-MD in that it only formed local lesions on the inoculated leaves of *N. tabacum* cv. 'Burley 21' plants (Table 3).

TABLE 1

The Frequency of TSWV Reassortants among Local Lesion Isolates from Plants Co-infected with TSWV-D and TSWV-10

Genotypes	Observed frequency	Expected frequency	χ^2	Significance
$L_DM_DS_D$(D)	15	7.25	8.28	
$L_{10}M_{10}S_{10}$(10)	3	7.25	2.49	
$L_{10}M_{10}S_D$(R1)	8	7.25	0.07	
$L_{10}M_DS_D$(R2)	31	7.25	77.8	
$L_DM_{10}S_D$(R3)	1	7.25	5.39	
$L_{10}M_DS_{10}$	0	7.25	7.25	
$L_DM_DS_{10}$	0	7.25	7.25	
$L_DM_{10}S_{10}$	0	7.25	7.25	
	58	58	115.78	$P < 0.001$

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10  TCTTGCTGTGTCCAGCCTTTTCTAATTATGTTATGTTTATTTTCTTTCTT
D   TCTTGCTGTGTCCGG-TTTTTCTAATTATGTTATGTTTATTTTCTTT-TT

10  TATTTATAATTATTTCTCTATTT-GTCATTTCTTTCAAATTCCTTCTGTC
D   TACTTATAATTATTTCTCTGTTTTGTCATTTCTTTTAAGTTCCTCCTGTT

10  TAGTAGAAACCATAAAAACAAAAA---TGAAAATAAAATAAATCAAAATA
D   TAATAGAAACCATAAAA-CAAAAATAAAATAAAAAT--AAAATCAAAAAT

10  AAAT--AAAATCAAAAATGAAAT----------AA
D   GAAACAAAAATCAAAAAATGAAACAAAAATAAAAAATAAAAAATGAAATA

10  AAAACAA------AAAATAAAAACAAAAAACCAAAAAAGATCCCGAAAGG
D   AAAACAACAAAAAAATTAAAAAACAGAAACCCAAAAAAGATCCCGAAAGG

10  -ACAATTTTGGCCAAATTTGGGTTTGTTTTTGTTGTT----TTTGTTTTT
D   GACAATCTTGGCCAAATTTGGGTTTTGTTTTGTTTTTGTTTTTTGTTTTT

10  TGTTTTTTGTTTTTATTTTATTTTATTTTATTTATTTTTATTTTATTTTT
D   TGTTTTTATTTTTATTTTATTTATTTATTTTATTTTATTTTT-ATTTTTT

10  GTTTTTATCTTATGTTT-----TTTTGTTGTTTTTGTTATTTTGTTA-TT
D   ATTTTGTTTTTTATTTTATGTTTTTTGTTGTTTTTGTTATTTTGTTT---

10  TATTAAGCACAACACACAGAAAGCAAACTTTATTTTATCTTATTTATTTT
D   --------------------------------------------------

10  TATTTTATTTTTGTTCTTATCTTATGTTTTTTGTTGTTTTTGTTATTTTA
D   ---------------------------------------------------

10  TTATTTATTAAGCACAACACACAGAAAGCAAACTTTAATTAAACACACTT
D   --ATTTATTAAGCATAACACACAGAAAGCAAACTTTAATTAAACACACTT


10  ATTTAAAATTTAACACACTAAGCAAGCACAAGCAATAAAGATAAAGAAAG
D   ATTTTAAATTTAACACACTAAGCAAGCACAAGCAATAAAGATAAAAAAAG

10  CTTTATATATTTATAGGCTTTTT-ATAATTTAACTTACAGCTGCTT 597nt
D   CTTTATATATTTGTAGACTTTTCCATAATTTAACTTACAGCTGATT 535nt
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FIG. 3. Alignment of the intergenic sequence (from nucleotide 1493 to 2027) of TSWV-D S RNA (GenBank Accession No. AF020660) and the intergenic sequence (from nucleotides 1493 to 2089) of TSWV-10 S RNA (GenBank Accession No. AF020659). The 33-nt duplication of TSWV-10 S RNA is underlined.

A novel viral phenotype capable of overcoming the resistance in *N. tabacum* cv. 'Burley 21N' mediated by the TSWV-D N gene was also observed (Table 3). The reassortant, $L_{10}M_{10}S_D$, systemically invaded the TSWV-derived resistant line. Resistance in this line was defined as the suppression of systemic invasion following local lesion production. Neither of the parental isolates, TSWV-D and TSWV-10, could systemically invade the TSWV-derived resistant plants although both caused symptoms on the inoculated leaves. Reassortants from three different experiments were tested on the resistant plants. In each experiment, only the $L_{10}M_{10}S_D$ genotype systemically invaded the resistant plants.

The phenotypes of reassortants with the same genotypes from separate reassortment experiments were shown to be consistent, and no other variability was observed. Reassortant phenotypes were also stable following transfer from *N. benthamiana* plants to *E. sonchifolia* plants and back to *N. benthamiana* plants.

DISCUSSION

Best (1961) studied the complexity of TSWV symptomatology and provided the first evidence for the possible reassortment or genetic recombination in TSWV. The system we have developed for genetic mapping of TSWV phenotypes consists of a group of genetically compatible isolates whose genome segments can be distinguished by a set of molecular markers used to authenticate the origin of each segment in the progeny reassortants. The genome segments from the parental isolates reassorted with relatively high frequency. We did not encounter the incompatibilities characteristic of viruses in the *Bunyavirus* genus (Pringle, 1996). Reassortment was restricted to viruses within the same serogroup in the genus *Bunyavirus,* and even then incompatibilities were observed (Pringle, 1996). Serogroups in the genus *Bunyavirus* are defined by the serological relatedness of the G1/G2

TABLE 2

Number of Nucleotides and Presence of the 33-nt Duplicate Sequence in S RNA IGR of TSWV Isolates from Diverse Geographical Locations

TSWV isolate	Geographical locations	Host plant	IGR length (nt)	Presence of 33-nt duplication
BRO1[a]	Brazil	Tomato	503	−
D	Netherlands	Dahlia	535	−
BL[b]	Hawaii	Lettuce	491	−
HR-2	Hawaii	Tomato	509	−
2A	Hawaii	Tomato	508	−
34	California	Chrysanthemum	500	−
35	California	Chrysanthemum	500	−
31	Texas	Peanut	621	+
32	Texas	Peanut	620	+
10	North Carolina	Peanut	597	+
8	North Carolina	Tomato	600	+
11	Georgia	Peanut	599	+
L3[c]	Bulgaria	N/A	585	+

[a] de Haan *et al.,* 1990.
[b] Pang *et al.,* 1992.
[c] Maiss *et al.,* 1991

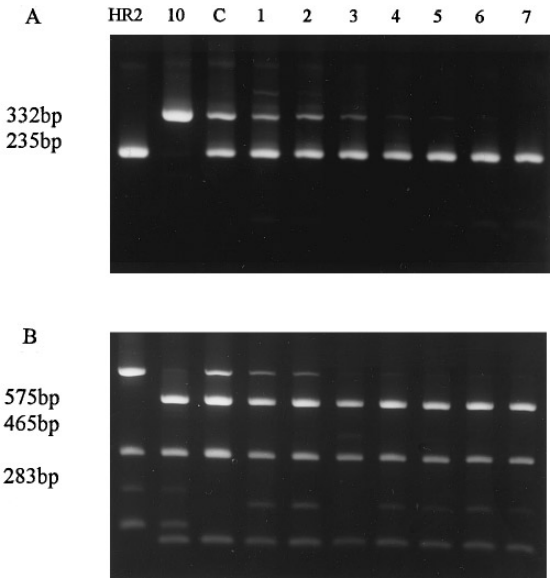

FIG. 4. Presence of detectable viral RNA segments during serial transfers of a viral mixture (TSWV-HR2 and TSWV-10) which reveal the competitive exclusion of the larger S RNA (TSWV-10) in the viral population. (A) A shift in the S RNA pool from a mixture of segments with short (TSWV-HR2) and long (TSWV-10) IGRs to the S RNA with short IGR following seven transfers (lane 1 to 7) on *N. benthamiana* plants. (B) The L RNA marker amplified from the same RNA extract which shows the dominance of TSWV-10 L RNA segment, supporting the concept that the competitive effect is segment specific. Lane C, Amplification from the reaction mixture containing equal amounts of TSWV-HR2 and TSWV-10 RNA template that demonstrates relative amplification efficiency.

proteins, whereas no separate taxa have been identified for tospoviruses based on the G1/G2 proteins.

Although we did not encounter incompatibility between isolates, individual genome segments from the specific isolates were preferentially acquired by some reassortants to the exclusion of the corresponding genome segment from the other parental isolate. The non-random segregation of genome segments has been reported in other viruses with segmented genomes, such as snowshoe hare and La Crosse bunyavirus (Urquidi and Bishop, 1992), subgroup 2 human rotavirus (Ward *et al.,* 1988), and type 1 Lang and type 3 Dearing reovirus (Nibert *et al.,* 1996). This phenomenon has been linked to the distinct replication efficiency of the two corresponding segments (Urquidi and Bishop, 1992), as well as the modulation of viral proteins from heterotypic segments in the newly formed reassortants (Nuttal *et al.,* 1992). The selective pressure of the host has also been shown to influence the pattern of reassortment (Graham *et al.,* 1987). In this study, there was a marked selective advantage for the S RNA from TSWV-D over TSWV-10. Then it was demonstrated that the S RNA from TSWV-10 was less competitive when paired against two other selected isolates, TSWV-HR2 (Fig. 4) and TSWV-34 (data not shown). The distinguishing characteristic of the less competitive S RNA segment was a longer intergenic region, which contained a 33-nt duplicate sequence embedded in a larger insertion sequence. This duplicate sequence was conserved among all TSWV isolates possessing the longer IGRs in the S RNAs. In another experiment, in which all six reassortants were isolated, neither parent contained the S RNA with the duplicate sequence in the IGRs (unpublished results).

There is a limited body of knowledge regarding the regulation of RNA replication in the family *Bunyaviridae.* Within this family, the least is known about viruses with an ambisense coding strategy for the M RNA or S RNA. It is known that the transcription termination sites for both transcripts from the S RNA of Punta Toro or Toscana *Phlebovirus* have been mapped to the IGR of the ambisense S RNA (Emery and Bishop, 1987; Gro *et al.,* 1992). Insertions analogous to those we identified have been reported in other viruses with ambisense genome

TABLE 3

Association of TSWV Phenotypes Expressed by Parental Isolates and Reassortants with Specific Genome Segments

Parental isolates	Genotypes	Phenotypes		
		Systemic infection on *N. tabacum cv.* 'Burley 21'	Systemic infection on transgenic *N. tabacum cv.* 'Burley 21N'	Local lesion diameter on *C. sativus* cotyledon[a]
TSWV-D	$L_DM_DS_D$	+	−	Small
+	$L_{10}M_{10}S_D$(R1)	+	+	Large
TSWV-10	$L_{10}M_DS_D$(R2)	+	−	Small
	$L_DM_{10}S_D$(R3)	+	−	Large
	$L_{10}M_{10}S_{10}$	+	−	Small
TSWV-D	$L_DM_DS_D$	+	−	Small
+	$L_DM_{MD}S_{MD}$	+	−	Small
TSWV-MD	$L_{MD}M_DS_{MD}$	−	−	Small
	$L_{MD}M_{MD}S_{MD}$	−	−	Small

[a] Local lesion diameter determined as average of 50 lesions from each isolate. The average diameter of the small lesions was 0.3 mm and of the large lesions was 1.2 mm.

segments including Rift Valley fever *Phlebovirus* and Sandfly fever Sicilian *Phlebovirus* (Giorgi *et al.,* 1991) and rice stripe *Tenuivirus* (Zhu *et al.,* 1992). These insertions and the repetitive sequences in the IGR have not been associated with any functions. This is the first report linking specific viral sequence in the IGR to a function which results in a competitive advantage for a genome segment.

In addition to demonstrating that the reassortment of genome segments occurs readily in TSWV, we mapped elements responsible for two viral phenotypes, local lesion morphology and systemic movement to specific genome segments. Little is known about the regulation of lesion morphology except that it mapped to the M RNA that codes for three proteins. Additional insight into the specific functions of ORFs will await a system to rescue infectious transcripts. In the reassortment from TSWV-D and TSWV-MD co-infection, two reassortants were isolated and the systemic movement phenotype segregated with the L RNA. However, additional reassortants are required to confirm the linkage. The putative association of systemic movement with the L RNA, which encodes the RNA-dependent RNA polymerase, is consistent with the results of investigation of long distance movement of some plus-sense RNA plant-infecting viruses (Nelson *et al.,* 1993; Petty *et al.,* 1990; Roossinck and Palukaitis, 1990). An unexpected result was the isolation of one reassortant that overcame the pathogen-derived resistance mediated by the N gene from TSWV-D. Since the resistance suppressed both parental viruses, this suggests that it impacts multiple viral functions mediated by viral proteins whose ORFs are on separate segments of the genome.

MATERIALS AND METHODS

TSWV isolates and phenotype determination

Three phenotypically distinct isolates, TSWV-D, TSWV-10, and TSWV-MD, were used in the reassortment study. TSWV-D, isolated from dahlia (*Dahlia* × *hybrida*) and originating in the Netherlands, has a distinct genotype (as described below), making it discernible from other isolates, but has a typical phenotype (Ie, 1970). TSWV-10 was isolated from peanut (*Arachis hypogaea*) in the USA. The TSWV-D and TSWV-10 isolates systemically infect *Nicotiana tabacum* cv. 'Burley 21' The TSWV-MD isolate only induces local lesions on *N. tabacum* cv. 'Burley 21' and is deficient for systemic movement. To reduce the genetic heterogeneity, each isolate was passaged through several single lesion transfers, in which a single lesion was excised, ground in inoculation buffer (10 mM Tris–HCl, pH 7.8, 10 mM Na_2SO_3, 0.1% cysteine-HCl), and transferred to the next set of plants. Viral stock cultures were maintained at −80°C and active viral cultures were maintained in *N. benthamiana* or *E. sonchifolia* plants.

Additional isolates of TSWV originating in California, Texas, Georgia, North Carolina, and Hawaii were used in the comparative analysis of the IGRs. TSWV-HR2 and TSWV-2A isolates were generously provided by Dr. John Cho, University of Hawaii. Those isolates were rescued from lyophilized or frozen tissues by transfer to *N. benthamiana* plants.

Parental isolate and reassortant phenotypes were characterized by symptom type and extent of invasion of plants of *C. annuum* (pepper), *C. sativus* cv. 'National Pickling' (cucumber), *E. sonchifolia, G. globosa, L. esculentum* (tomato), *N. glutinosa, N. benthamiana, N. tabacum* cv. 'Burley 21,' and *N. tabacum* cv. 'Burley 21' transformed with the TSWV-D N gene (Burley 21N). Three host phenotypes were selected for scoring viral phenotypes in parental and reassortant isolates: (i) lesion diameter on cucumber cotyledons, (ii) systemic infection of *N. tabacum* cv. 'Burley 21,' and (iii) the ability to overcome the suppression of systemic invasion in resistant *N. tabacum* cv. 'Burley 21' conferred by the TSWV-D N transgene. Lesion diameter on cucumber cotyledons was determined by averaging the diameter of 50 lesions in three replicate experiments. The lesions induced by TSWV-D and TSWV-10 were of two nonoverlapping populations classified as large or small, respectively, with mean diameters of 0.3 or 1.2 mm. Detection of the systemic invasion of *N. tabacum* cv. 'Burley 21' plants was based on symptom expression on the upper noninoculated leaves.

Generation of reassortants

Reassortants were generated by amplifying inocula in *N. benthamiana* plants and then selection through multiple local lesion transfers on *N. tabacum* cv. 'Burley 21' plants. Systemically infected leaves of *N. benthamiana* co-inoculated with TSWV-D and TSWV-10, or TSWV-D and TSWV-MD, were used to inoculate *N. tabacum cv.* 'Burley 21' plants. To increase the probability of generating the six theoretically possible reassortants from each pair of parental isolates, a range of ratios of the two inocula prepared from leaf tissue infected by the parental isolates was used in the initial co-infection. Single lesions with an array of varying morphologies and latent periods were transferred to *N. tabacum* cv. 'Burley 21' plants to increase the probability of recovering the six possible reassortants. Local lesions from the inoculated leaf were excised and ground in inoculation buffer and transferred to *N. tabacum* cv. 'Burley 21' plants. Each local lesion isolate was serially transferred a minimum of three times or until the lesion type was uniform in an effort to reduce the probability of the isolate being a mixture of more than one TSWV genotype. Following the final transfer, one lesion was ground in 200-μl inoculation buffer and inoculated onto both *N. benthamiana* plants from which viral RNA template was isolated for RT-PCR and *N. tabacum* cv. 'Burley 21' plants for maintenance.

Genetic markers and genotyping of reassortants

TSWV virions were purified from systemically infected *N. benthamiana* leaves as described by Law and Moyer (1990). Virion RNAs were extracted as described by Moyer and Cali (1985). Total plant RNA was extracted from 100 mg of TSWV-infected *N. benthamiana* leaf tissue using RNA ISOLATER (Genosys, Woodlands, TX) according to the supplier's protocols.

The genome segments from each pair of TSWV isolates were distinguished by RFLPs of RT-PCR amplified cDNA synthesized from L, M, and S RNAs. Two regions were amplified from each genome segment to detect potential recombination of major regions on that segment. Two reactions were carried out on each sample: reaction one with L4856, M4037, and S2767 as cDNA synthesis primers and reaction two with L28, M962, and S890 as cDNA synthesis primers (Fig. 1) under conditions specified for AMV reverse transcriptase (Promega, Madison, WI). The first strand cDNA was extracted by phenol:chloroform and precipitated in 0.5 vol of 7.5 M ammonium acetate and 2.5 vol of 95% ethanol. Air-dried cDNA was resuspended in 10 μl sterilized water. Individual PCR were carried out for the L, M, and S segments by mixing 2 μl cDNA with 0.4 μM of the viral complementary sense primers and 0.4 μM of the viral sense primers. The final reaction mixture contained 10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 μl/ml gelatine, pH 8.3, 2 units *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN), 250 μM dNTPs (each). Amplified products were analyzed following forty cycles of amplification, with each cycle consisting of 94°C, 1 min, 55°C, 1 min, 72°C, 2 min, with 94°C, 10 min at the beginning and 72°C, 10 min at the final step.

The origin of each genome segment (TSWV-D, TSWV-10, or TSWV-MD) in the progeny isolates was determined from the RFLP of the RT-PCR-generated fragments. The amplified L RNA (nt 4856 to 5578), M RNA (nt 3050 to 4037), and S RNA (nt 1983 to 2767) fragments were digested with *Alu*I. The amplified L RNA (nt 28 to 895), M RNA (nt 66 to 962), and S RNA (nt 70 to 890) fragments were digested by *Taq*I, *Rsa*I, and *Taq*I, respectively.

The genetic marker for typing S RNA fragments in the competition assay was later replaced by resolving the length difference of the RT-PCR-amplified central regions of the IGRs through agarose gel electrophoresis. The viral complementary sense primer, SIGR1 (Fig. 1), was used to perform the first strand cDNA synthesis of the IGR and to amplify the partial IGR region directly from total RNA with the viral sense primer SIGR2 (Fig. 1).

Determination of 5′ and 3′ termini of TSWV S RNA

A strategy for determining the 5′ and 3′ termini of viral RNA by ligation-anchored PCR and anchored cDNA cloning (Weng and Xiong, 1995) was adopted to determine the authentic 5′ and 3′ terminal sequences of TSWV S RNA. The primers, S156R and S2500, were based on the TSWV-D S RNA sequence. Primers HNER and HNEF contained *Hin*dIII, *Not*I, *Eco*RV restriction enzyme sites (Fig. 1). The resulting 5′ and 3′ terminal cDNAs were inserted into pGEM-T vectors according to the supplier's protocol (Promega Co., Madison, WI).

Full-length cDNA synthesis of S RNA, cloning, and sequencing

The full-length cDNA of the S RNA was synthesized from the virion RNA template by RT-PCR. PCR was performed by placing 2 μl cDNA in 50 μl reaction solution containing 20 mM Tris–HCl, pH 9.2, 60 mM KCl, 2 mM $MgCl_2$, 0.25 mM dNTP (each), 0.4 μM of the viral complementary sense primer (CGGGA TCCTC TAGAG CAATC GTGTC AATTT TGTGT TCATA CCTTA ACACT C, viral sequence underlined) possessing *Bam*HI and *Xba*I restriction sites, and the viral sense primer (CGGGA TCC*TA ATACGA CTCAC TATA*G GAGAG CAATT GTGTC AGAAT TTTGT TC) having the *Bam*HI restriction site and T7 promoter sequence (italics), with 5 units *TaqPlus* (Stratagene Co., La Jolla, CA). The reaction mixture was covered by 65 μl sterilized light mineral oil. The amplification cycle consisted of denaturing at 94°C, 2 min; annealing at 55°C, 1 min; extension at 72°C, 3 min; 30 cycles of 94°C, 30 s; 55°C, 1 min; 72°C, 3 min; and final extension at 72°C, 5 min.

RT-PCR-amplified viral cDNAs were cloned into pGEM-T vectors (Promega, Madison, WI). The cloned cDNAs were sequenced by the dideoxy-nucleotide chain termination method (Sanger *et al.,* 1977) using sequenase 2.0 (US Biochemicals, Cleveland, OH). All nucleotide sequences were verified by sequencing two separate clones. The S RNA IGR sequences were determined and corroborated by sequencing the IGR cDNA clones. First strand cDNAs of the viral S RNA IGRs were synthesized from total RNA and initiated by the viral complementary sense oligonucleotide primer S2083 (Fig. 1). The resultant cDNA was subjected to PCR amplification initiated by oligonucleotide S2083 and the viral sense oligonucleotide primers S1457 or S1282 (Fig. 1).

S RNA genome segment competition assay

The isolate pairs [TSWV-HR2 (short S RNA) and TSWV-10 (long S RNA)] and [TSWV-34 (short S RNA) and TSWV-10] were co-inoculated onto *N. benthamiana* or *N. tabacum* cv. 'Burley 21' plants. The resultant mixed viral population was subjected to repeated serial passages on 'Burley 21' plants. The S RNA marker residing within the IGR was used to type the S RNA from each isolate. The L RNA marker for differentiating isolates was used as an internal control to determine if the associated segments were maintained at similar concentrations throughout the serial transfers.

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